

EXHIBIT A

Review

Vasoprotection by nitric oxide: mechanisms and therapeutic potential

Michael T. Gewaltig, Georg Kojda*

*Institut für Pharmakologie und Klinische Pharmakologie, Medizinische Einrichtungen, Heinrich-Heine-Universität, Moorenstrasse 5,
40225 Düsseldorf, Germany*

Received 7 November 2001; accepted 14 February 2002

Abstract

Endothelial production of nitric oxide (nitrogen monoxide, NO) has become a major research area in vascular biology. Some of the most important effects that NO exerts in the vascular wall are potentially vasoprotective, because these effects maintain important physiological functions such as vasodilation, anticoagulation, leucocyte adhesion, smooth muscle proliferation, and the antioxidative capacity. During the last 2 decades it has become apparent that a variety of diseases are associated with an impairment of endothelium-dependent NO activity. One of the major causes is believed to be an increased production of reactive oxygen species, in particular superoxide, which have been shown to interfere with many steps of the NO–cyclic guanosine monophosphate (cGMP) pathway. This phenomenon has been found in diverse conditions such as atherosclerosis, hypertension, diabetes, hypercholesterolemia, heart failure, and cigarette smoking. The aim of this review is to examine the cellular and molecular mechanisms whereby NO exerts potentially vasoprotective effects and to discuss pharmacologic approaches targeting the NO pathway in view of their potential to improve endothelial function and to reduce the progression of atherosclerotic vascular disease. We conclude that there is compelling evidence for vasoprotective actions of NO which are mediated by cGMP-dependent and cGMP-independent mechanisms. These effects may contribute to the beneficial effects of established drugs such as ACE inhibitors or statins. Unfortunately, clinical data on the effect of long-term treatment with nitrates on the progression of coronary artery disease are lacking. Finally, L-arginine or new activators of the NO pathway may become therapeutic options in the future.

© 2002 Elsevier Science B.V. All rights reserved.

Keywords: Atherosclerosis; Endothelial function; Nitric oxide; Vasoactive agents

1. Introduction

Only 6 years after the discovery of endothelial-derived relaxing factor (EDRF) it became apparent that many cardiovascular diseases are associated with an impairment of endothelium-dependent vasorelaxation. This has been shown in hypercholesterolemic rabbits and monkeys and in patients having coronary artery disease or typical risk factors predisposing to this condition. Endothelium-dependent vasorelaxation is also abnormal in other disease states such as heart failure, diabetes and hypertension [1].

Presumably, there is a loss of endothelial production and/or bioavailability of NO (nitric oxide, nitrogen monoxide) in these disorders. The term ‘endothelial dysfunction’ evolved in the scientific literature in order to conveniently label the above mentioned alteration of vascular endothelial function. Although this term is somewhat imprecise, it has become widely used. Endothelial dysfunction may refer to impairment of important endothelial functions including anticoagulant and antiinflammatory properties [2].

The mechanisms underlying altered endothelium-dependent vascular relaxation in various disease states are multifactorial and have been reviewed previously [3].

*Corresponding author. Tel.: +49-211-81-12518; fax: +49-211-81-14781.

E-mail address: kojda@uni-duesseldorf.de (G. Kojda).

Time for primary review 27 days.

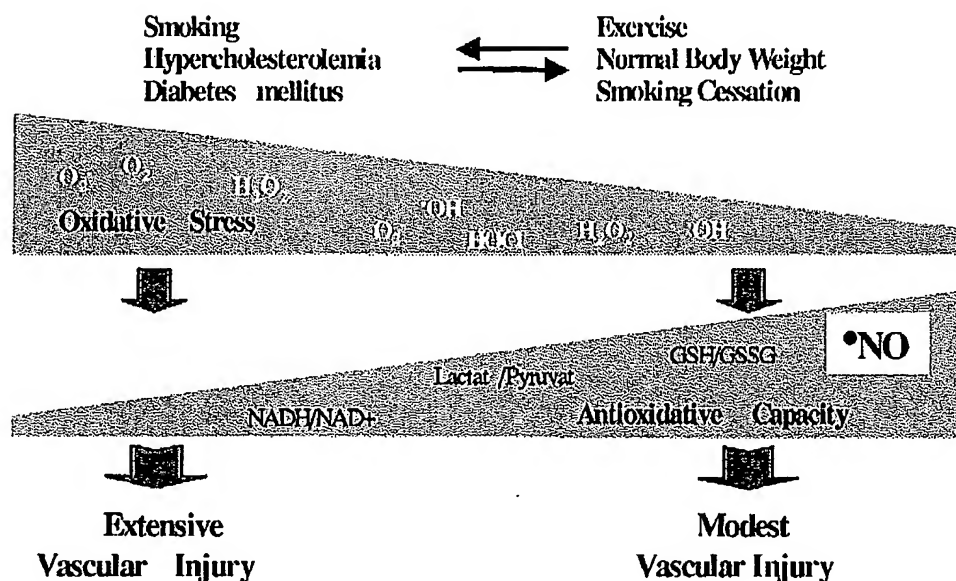


Fig. 1. Changes of the balance between oxidative stress and antioxidative capacity in the vascular wall as induced by typical risk factors or interventions known to reduce the incidence of cardiovascular events.

Many typical cardiovascular risk factors are associated with an increase of vascular oxidative stress and/or accompanied by a decrease of the antioxidative capacity of the vascular wall (Fig. 1). It has been shown recently that the degree of endothelial dysfunction is most likely associated with the risk of a cardiovascular events during a mean follow-up period of 6.7 years [4]. These interesting results suggest that attenuation of endothelial dysfunction should reduce the rate of cardiovascular events and improve the prognosis of patients.

2. NO-cGMP pathway

The pharmacology of guanylyl cyclases and cellular actions of cGMP has been reviewed recently [5]. Briefly, NO is the only known endogenous formed radical acting as a signaling messenger. It is formed by NO synthases (NOSs), which convert L-arginine to citrulline and NO. NOSs are widely distributed throughout the body and have various important physiologic functions. These include the regulation of blood pressure, local vasomotion and sexual functions (penile erection and ejaculation), processing of long-term potentiation in the central nervous system and a contribution to the immune defense [1,6–8]. Within the cardiovascular system the most important NOS isoform is endothelial NOS (NOSIII, eNOS, eNOS). In endothelial cells this enzyme is bound to cell membrane associated caveolae. The major physiologic stimulus of vascular NO production is the blood flow-induced shear force (shear stress) on endothelial cells [9]. Shear stress induced eNOS

activation is most likely not mainly mediated by a rise in intracellular calcium but is directly dependent on phosphorylation by serine/threonine protein kinase Akt/PKB [10]. A recent report suggests that receptor-mediated activation of eNOS by agonists such as acetylcholine (which increases intracellular calcium) is also dependent on phosphorylation by Akt [11].

NO activates the enzyme soluble guanylate cyclase (sGC) to produce the second messenger cyclic guanosine monophosphate (cGMP, Fig. 2). Activation of this enzyme is dependent on binding of NO to the heme moiety of sGC to form the nitrosyl-heme adduct of sGC. As a consequence, the heme iron is shifted out of the plane of the porphyrine ring configuration which initiates the binding of GTP and the formation of cGMP [12]. Cyclic GMP activates two specific cGMP-dependent protein kinases (PKG I and PKG II) of which PKGI is the major kinase mediating vasodilation and inhibition of platelet aggregation [13,14].

The activity of cGMP is terminated by rapid conversion to GMP which is catalyzed by various phosphodiesterases (PDE). Of these, PDE V is particularly specific for cGMP [15]. Cyclic GMP can also regulate the activity of some PDE isoforms such as the cGMP-inhibited PDE (PDE III) and the cGMP-activated PDE (PDE II). While the inhibition of PDE III elevates intracellular cAMP concentrations and stimulates protein kinase A (PKA) activity, cGMP-dependent activation of PDE II has the opposite effect. Therefore, NO might exert vasoprotective actions via both cyclic nucleotide protein kinases, but stimulation of mainly PKG I is probably more important [14].

3. Potentially vasoprotective mechanisms of NO

3.1. Vasodilator effects of NO

Vasodilation is the best documented activity of NO in the cardiovascular system. This action led to the discovery of endothelium-derived relaxing factor 20 years ago [16]. Subsequent research has shown that endogenous NO production is involved in the regulation of local vasomotion and blood pressure. Numerous conditions characterized by an impaired availability of NO have been found to be associated with enhanced synthesis of ET-1 as a potent endogenous vasoconstrictor [17]. Pharmacological inhibition of endogenous NO synthesis has been shown to induce a rise in blood pressure in man and disruption of the eNOS gene by the 'knock-out' technique causes mild hypertension in mice [18,19]. Elevated blood pressure is a well known risk factor for the development of cardiovascular diseases such as stroke and myocardial infarction, while a reduction of blood pressure is effective in reducing morbidity and mortality of cardiovascular diseases [20]. Thus, maintenance of normal blood pressure by endothelial NO may be considered as part of its vasoprotective action.

The mechanism underlying the NO-induced vasodilation has been intensively investigated. Current knowledge suggests a central role for cGMP-dependent activation of PKG I which can phosphorylate different membrane proteins in the sarcoplasmic reticulum (Fig. 2). It has been reported that PKG I can phosphorylate phospholamban [21]. In its dephosphorylated state, phospholamban monomers inhibit the sarcoplasmic reticulum ATPase (SERCA) by binding to its cytoplasmic and membrane domains causing Ca^{2+} pump aggregation. Phosphorylation of phospholamban (e.g. by PKG I) favors the association of phospholamban monomers into pentamers and reverses the inhibition of SERCA [22]. This initiates a rapid sequestration of intracellular calcium, which in turn also reduces the influx of extracellular Ca^{2+} into the sarcoplasmic reticulum [23].

Another more recent report demonstrates that activation of the PKG I phosphorylates a newly-discovered protein, the 1,4,5-inositoltrisphosphate (IP_3) receptor associated cGMP kinase substrate (IRAG) [24]. Phosphorylation of IRAG results in a strong inhibition of IP_3 -evoked Ca^{2+} release from the sarcoplasmic reticulum. Currently, it is not known how IRAG phosphorylated by PKG I interacts with the IP_3 receptor.

NO can also activate Ca^{2+} -dependent K^+ channels and increase the outward potassium current [25]. The resulting hyperpolarization of the cell membrane decreases the effect of the depolarizing signals and induces vasodilation. It has been shown that this action of NO can be both independent and dependent on activation of PKG I [25,26]. Finally, cGMP-dependent inhibition of voltage-gated calcium channels might also be involved in the mechanism of vasodilation induced by NO [27]. The relative contribution

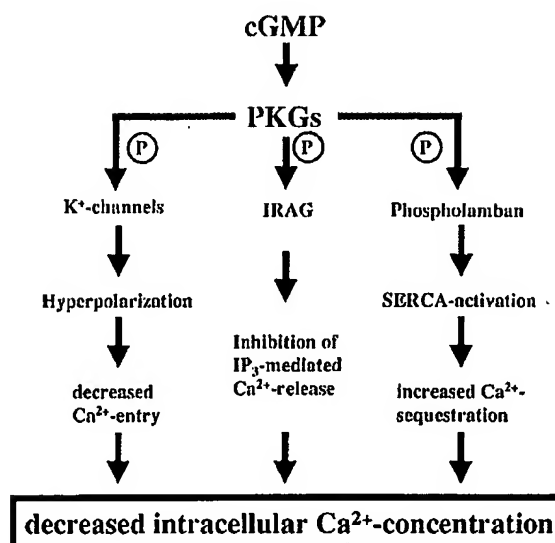


Fig. 2. Mechanisms of vasorelaxation induced by NO. After the activation of soluble guanylyl cyclase, cGMP is formed which in turn stimulates cGMP-dependent protein kinases (PKGs). Subsequently, three important proteins are phosphorylated resulting in a decrease of the intracellular Ca^{2+} concentration. The direct effect of NO on K^+ -channels and the cGMP-dependent inhibition of voltage-gated calcium channels is not shown; K^+ -channels, Ca^{2+} -dependent potassium channels; IP_3 , 1,4,5-inositol trisphosphate; IRAG, IP_3 receptor associated cGMP kinase substrate; SERCA, sarcoplasmic reticulum ATPase.

of each of these PKG I- and K^+ -channel-dependent vasodilating mechanisms of NO remains to be determined. The subsequent reduction of the intracellular Ca^{2+} concentration reduces the formation of the Ca^{2+} -calmodulin-myosin light chain kinase complex. This decreases phosphorylation of Ser19 in the myosin regulatory light chains and inhibits vasoconstriction [28].

3.2. Antiplatelet effects of NO

Platelets play a vital role in vascular haemostasis. Their ability to aggregate and form a haemostatic plug must be carefully balanced against the necessity to maintain the fluid state of the blood and to avoid thrombosis [29]. Atherosclerotic changes are often followed by platelet hyperactivity associated with thrombosis, myocardial infarction and stroke. NO and NO donors stimulate cGMP production in human platelets leading to activation of PKG and inhibition of platelet aggregation induced by agonists (e.g. thrombin which increase the intracellular Ca^{2+} concentration) [30,31]. It is known that NO-induced inhibition of platelet aggregation involves a decrease of the intraplatelet Ca^{2+} concentration [32].

Similar to the mechanism of NO-induced vasorelaxation (Fig. 2), NO-induced inhibition of platelet aggregation

involves phospholamban- and SERCA-dependent refilling of intracellular Ca^{2+} stores [33,34]. In addition, cGMP has been shown to indirectly activate PKA, since cGMP inhibits the breakdown of cAMP by PDE III [35]. Both nucleotides are known to phosphorylate phospholamban, which then activates SERCA to enhance the sequestration of Ca^{2+} [36,37]. NO and cGMP analogues act synergistically with cAMP-elevating agents such as prostacyclin to inhibit platelet aggregation [35]. In summary, both cAMP and cGMP-elevating agents inhibit platelet aggregation by a reduction of the intracellular Ca^{2+} concentration.

3.3. Antiadhesive effects of NO

Increased leucocyte adhesion is a major step in the pathogenesis of atherosclerosis [38]. Leucocyte adhesion is critically dependent on the expression and appearance of various adhesion molecules on the cell surface of vascular endothelial cells. Most likely, increased expression of adhesion molecules such as vascular cell adhesion molecule (VCAM-1), intercellular cell adhesion molecule (ICAM), monocyte chemoattractant protein 1 (MCP-1) and E-selectin as well as various chemoattractant proteins (cytokines) is a consequence of increased vascular oxidant stress [39]. Activation of redox sensitive transcription factors such as nuclear factor κB (NF κB) and activating protein 1 (AP-1) is believed to play a key role in this process [40]. Monocytes which adhere to vascular cells migrate into the vascular wall and further increase oxidant stress by releasing large amounts of reactive oxygen species (ROS) [41]. Thus, a reduction of adhesion molecule expression or inhibition of adhesion molecule function are considered as vasoprotective strategies reducing the progression of vascular damage.

NO is an important endogenous mediator which inhibits leucocyte adhesion [42]. NO released by NO donors has been shown to potently inhibit the expression of VCAM-1 [43], while the inhibition of endogenous NO synthesis had the opposite effect [44]. Khan et al. provided evidence that this action of NO is mediated through inhibition of NF κB expression and involves antioxidative properties of NO. Oxidation of polyunsaturated fatty acids such as linoleic acid to peroxidized metabolites has been shown to be an important intermediate step in cytokine-induced activation of the redox-sensitive transcription factor NF κB and this oxidation can be markedly reduced by exogenous NO [43]. Likewise, increased leucocyte adhesion induced by the inhibition of NO synthases was at least partially reversed by various intracellular oxygen radical scavengers [44]. Thus, the mechanism of the antiadhesive action of NO most likely involves antioxidative effects (see below). In contrast, activation of PKG by cGMP does not seem to be involved, since cell permeable cGMP-analogs—unlike oxygen radical scavengers—cannot reduce leucocyte adhesion stimulated by NOS inhibition [44].

3.4. Antiproliferative effects of NO

Proliferation of smooth muscle cells plays a key role in the narrowing the lumen of blood vessels in coronary artery disease and restenosis [41,45]. In this process vascular smooth muscle cells show substantial changes of their function such as the disappearance of the contractile activity. These changes include a loss of myofibrils and the generation of matrix proteins. Furthermore, the susceptibility to proliferating stimuli such as platelet-derived growth factor (PDGF) enhances. These proliferating smooth muscle cells can migrate into the intima and contribute to intimal hyperplasia. Both proliferation and migration are controlled by various signaling molecules such as angiotensin II, tumor necrosis factor α (TNF α) and several growth factors (TGF- β , FGF, PGDF). The mechanisms of vascular smooth muscle growth have been reviewed recently [46].

NO has been shown to inhibit smooth muscle proliferation. This holds true for both NO generated by the vascular endothelium [47] and NO generated by NO donors [48]. Likewise, balloon angioplasty induced a much more pronounced intimal hyperplasia in mice lacking the endothelial NOS as compared to normal mice [49]. The mechanism underlying the antiproliferative activity of NO is not yet fully understood (Fig. 3). It has been suggested that cGMP-dependent activation of the PKA, partially mediated by the inhibition of the cGMP-inhibited-cAMP-phosphodiesterase (PDE III) may contribute [50,51]. A similar pathway of activation of PKA was reported to potentiate the NO-induced inhibition of platelet aggregation and to mediate the positive inotropic effects of NO [35,52]. It was suggested recently that NO-dependent activation of PKA can regulate the expression of cell cycle proteins [53]. Activation of PKA is thought to reduce smooth muscle proliferation by inhibition of Raf-1 [54].

Another potentially important mechanism underlying the antiproliferative effects of NO, which occurs independently of cGMP generation, is the inhibition of arginase and ornithine decarboxylase [55]. Experiments with rat aortic smooth muscle cells demonstrated that antiproliferative concentrations of NO donors strongly decreased the cellular content of polyamines such as putrescine, spermidine and spermine and inhibited the activity of purified ornithine decarboxylase. Although the NO donors effectively increased the cellular cGMP content, its modulation by inhibition of sGC or PDEs had no effect on cell proliferation. In contrast, inhibition of cell proliferation by NO donors was effectively reversed by putrescine but not by ornithine.

3.5. Antioxidative effects of NO

Vascular oxidative stress contributes to the pathophysiology of cardiovascular diseases. Among the various reactive oxygen species formed under these conditions superoxide

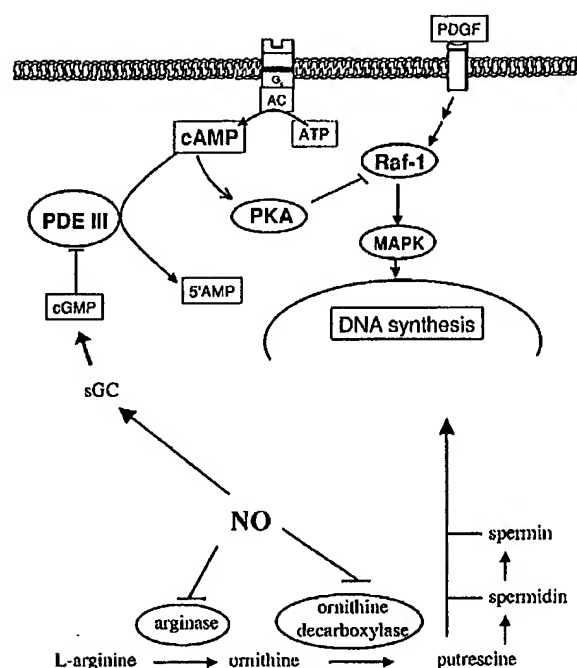


Fig. 3. Inhibition of PDGF-induced mitogenesis by cAMP–PKA. Activation of PKA can be mediated by the cGMP-induced inhibition of PDE III and has been shown to be important for the antiproliferative effects of NO. Another important but cGMP-independent mechanism of inhibition of cell proliferation by NO is mediated by inhibition of arginase (I and II) and ornithine decarboxylase (activation of PKA by direct cGMP-dependent PKA stimulation is not shown; PKA, cAMP-dependent protein kinase; AC, adenylyl cyclase; PDE III, cGMP-inhibited cAMP phosphodiesterase; sGC, soluble guanylyl cyclase).

is presumably the most important one [3]. The complex interactions of oxygen species with vascular signaling systems have been recently reviewed [56]. The best known antioxidative effect of NO is the impairment of lipid oxidation. NO can potently inhibit the oxidation of free fatty acids, phosphatidylcholine and low density lipoprotein particles. In view of the proatherogenic effects of oxidized lipids, this antioxidative activity of NO is likely to be relevant [57].

NO reacts very rapidly with superoxide to form peroxynitrite which is a much stronger oxidant than superoxide itself. In vivo, peroxynitrite rapidly forms a carbonate adduct (ONOOCCO_2^-) or oxidizes free undissociated thiols [58]. The carbonate adduct can rapidly form carbonate and nitrogen dioxide radicals [59]. Recently, these radicals have been shown to oxidize thiols to thiyl, sulfinyl and disulfide radicals [60]. The oxidizing potential of peroxynitrite and the subsequently formed radicals facilitate lipid peroxidation, induce protein damage by tyrosine nitration, dityrosine formation and thiol oxidation and reduce the antioxidative capacity of vascular cells by the rapid oxidation of free undissociated thiols [60–62].

In view of these reactions, it seems rather questionable that the increased generation of NO in the setting of oxidative stress might be associated with antioxidative properties or might increase the antioxidative capacity of the vascular wall. However, the formation of peroxynitrite from superoxide competes with the formation of hydrogen peroxide catalyzed by superoxide dismutases (Fig. 4). Although superoxide reacts approximately 3–6 times faster with NO than with superoxide dismutase, the formation of ONOO^- is outcompeted if the concentration of superoxide

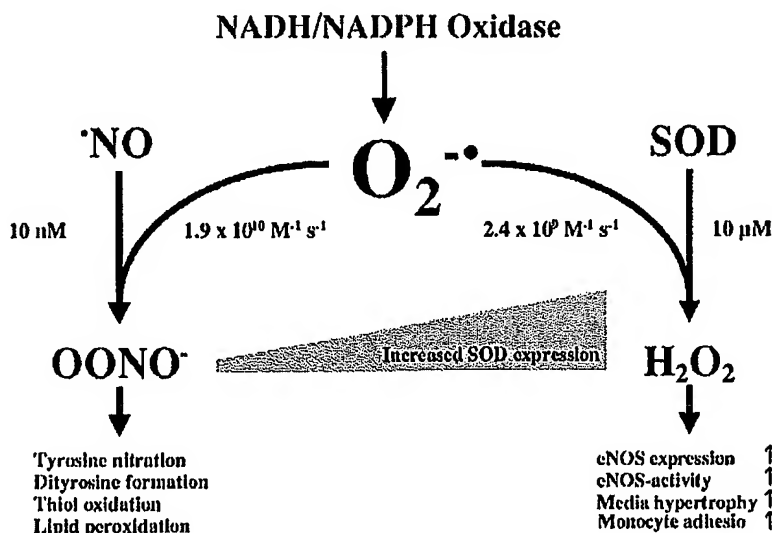


Fig. 4. Reactions of superoxide with SOD and NO. The reaction constants and the mean concentrations of intracellular and membrane associated SOD and of NO in the vascular wall suggest that the formation of hydrogen peroxide is favored. In addition, an increase of SOD expression as induced by exercise and NO would further shift the balance to hydrogen peroxide formation. The role of hydrogen peroxide as a vascular oxidant is unclear. Both, vasoprotective actions and proatherosclerotic actions have been reported.

dismutase is much higher than that of NO, a situation that is usually found in the intracellular space of vascular endothelial and smooth muscle cells [58]. Thus, physiologic concentrations of vascular NO, as generated by eNOS or by therapeutic interventions, might exert antioxidative effects in the presence of physiologic concentrations of superoxide dismutases [63]. In contrast, very high concentrations of NO, as generated by iNOS, presumably promote peroxynitrite formation.

NO has been identified as an inducer of endothelial ferritin synthesis [64,65]. Ferritin binds free iron ions and may reduce oxidative cellular damage by preventing superoxide generation [66]. Translation of ferritin mRNA is dependent on free iron ions as generated by the activity of heme oxygenase which converts heme into biliverdin. It has been shown that NO induces heme oxygenase-I expression suggesting that this effect is involved in the induction of ferritin by NO [67]. Another consequence of increased heme oxygenase-I expression is the increased formation of bilirubin and carbon monoxide [68]. Bilirubin can act as a scavenger of superoxide and carbon monoxide and is known to activate sGC [69]. Interestingly, a recent study in atherosclerotic mice showed that an increased expression of heme oxygenase-I can reduce the formation of atherosclerotic lesions [70]. These data suggest that the antioxidative effects of physiologic concentrations of NO might be mediated by induction of heme oxygenase-I and ferritin expression.

Another antioxidative effect of NO is via the induction of extracellular superoxide dismutase (ecSOD) which has been shown to occur *in vitro* and *in vivo* [71]. This potent antioxidative enzyme is expressed in vascular smooth

muscle cells and located at the outer cell membrane. In the vascular wall one third to one half of the total superoxide dismutase is the extracellular type of the enzyme [72]. The upregulation of extracellular superoxide dismutase expression in vascular smooth muscle cells may represent an important mechanism that prevents superoxide mediated degradation of endothelial NO as it traverses between the two cell types. Likewise, the formation of peroxynitrite will be reduced, because higher amounts of superoxide dismutase favors the dismutation of superoxide to hydrogen peroxide [58]. Recently, it was reported that just a brief exposure of endothelial cells to the strong oxidant hydrogen peroxide can increase the expression and activity of eNOS [73], a mechanism that might contribute to the antioxidative effects of NO.

Increased expression of ecSOD is also a vascular adaption to exercise. Normal mice undergoing 3 weeks of exercise showed an increase of vascular ecSOD expression, while the expression of Cu/Zn-SOD was unchanged [71]. In contrast, mice homozygous for a lack of the eNOS gene showed no increase of ecSOD expression suggesting that this effect of exercise was almost exclusively mediated by an increase of endogenous NO production. Thus, exercise training most likely increases the antioxidative capacity of the vascular wall by increasing the expression of the two functionally related proteins eNOS and ecSOD (Fig. 5). Of note, experiments with mice heterozygotic for a loss of the eNOS gene suggest this mechanism requires both alleles of the eNOS gene [74].

Taken together, data of several studies suggest that the antioxidative effects of NO are at least in part mediated by the induction of the expression of heme oxygenase-I,

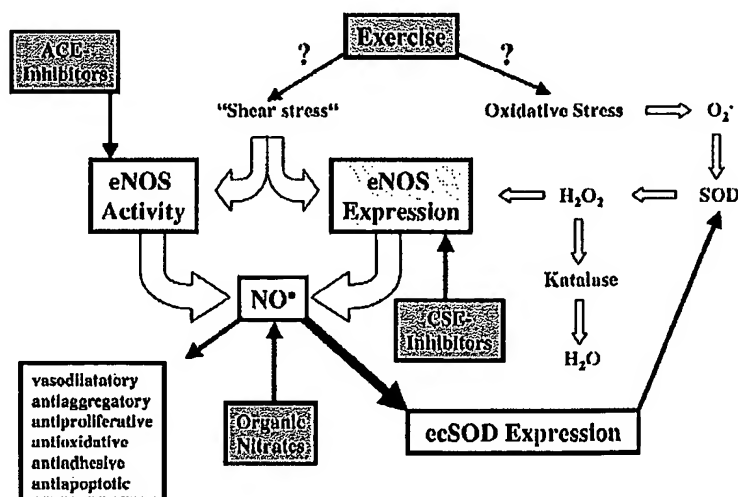


Fig. 5. Putative effects of exercise in the vascular wall. Exercise induces both, shear stress and oxidative stress. As indicated, the relative contribution of each effect to the increase of eNOS expression is not known. There is evidence that exercise increases the formation of NO in the vascular wall. This effect is most likely an important vascular adaptation to exercise and presumably contributes to the beneficial effect of physical activity in cardiovascular patients [111]. Some cardiovascular drugs are also known to increase vascular NO production (ACE, angiotensin converting enzyme; CSE, cholesterol synthesizing enzyme).

ferritin and extracellular superoxide dismutase. These activities of NO decrease both the superoxide levels and the formation of peroxynitrite in the vascular wall.

4. Drugs targeting the NO signaling pathway

4.1. Organic nitrates

Organic nitrates deliver NO directly to the vascular wall. These drugs are enzymatically denitrated to give NO and the denitrated metabolites [75]. The enzymatic conversion involves a transfer of three electrons to the nitrate nitrogen and is most likely mediated by cytochrome P 450 type enzymes such as CYP3A4 [76]. Generation of NO from organic nitrates occurs in both vascular endothelial and smooth muscle cells. Therapeutic concentrations of organic nitrates dilate preferentially venous vessels and conduit arteries such as the left anterior descending artery, while higher doses also decrease peripheral resistance [75].

Oral administration of the long acting organic nitrate pentaerythritol tetranitrate (PETN) to rabbits fed a high cholesterol diet for 4 months reduced the formation of aortic lesions and prevented the inhibition of endothelium-dependent vasodilation [77]. This protective effect of PETN could be confirmed in a subsequent study where the oral administration of PETN was initiated after the induction of atherosclerosis by feeding rabbits a cholesterol chow for 4 months (Fig. 6) [78]. In addition, PETN significantly reduced the oxidation of low density lipoprotein particles. Isosorbide mononitrate also reduced endothelial dysfunction and decreased the intimal lesion area and intimal thickness in the hypercholesterolemic rabbit thoracic aorta [79]. In contrast, a study with molsidomine, a prodrug being metabolized to the NO and superoxide releasing compound SIN-1, showed an augmentation of aortic intimal lesion in cholesterol fed rabbits [80]. However, this might have been caused by a proatherosclerotic effect of peroxynitrite rather than NO.

Importantly, there is no clinical evidence for the antiatherosclerotic effects of NO donors or organic nitrates in stable coronary artery disease. A meta-analysis summarizing earlier trials with nitrates revealed a total reduction of mortality from acute myocardial infarction of as much as 35% [81]. Most of the beneficial effects of nitrates have been attributed to the initial 24–48 h of therapy. This favorable result could not be confirmed in two recent mega-trials including 77 000 postinfarction patients [82,83]. In these studies, therapy with a glyceryl trinitrate patch (GISSI-3) or with an oral sustained formulation isosorbide mononitrate (ISIS-4) was initiated in the early postinfarction phase and continued for 5–6 weeks. Overall, only a trend but not a significant improvement in survival was observed. In contrast, subgroup analysis showed a significant benefit of nitrates on survival of women and patients older than 70 years and the combination of the

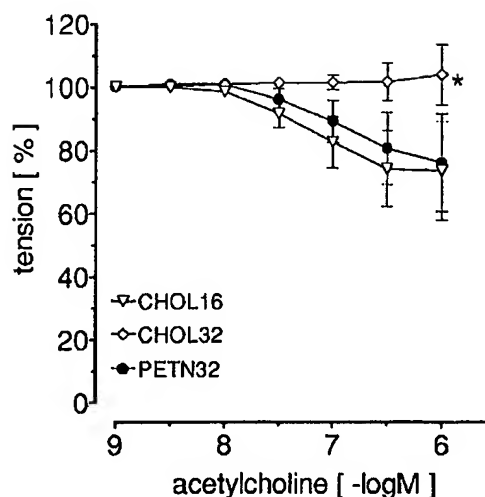


Fig. 6. Effect of the organic nitrate pentaerythritol tetranitrate (PETN) on the progression of endothelial dysfunction in established atherosclerosis in cholesterol-fed rabbits. PETN was added to the cholesterol chow at week 16 and given for 16 weeks. The two other groups received the cholesterol chow only for 16 and for 32 weeks. There was a considerable progression of inhibition of acetylcholine-induced aortic relaxation, when the cholesterol chow was given for 32 weeks (CHOL32) as compared to the 16-week feeding period (CHOL16). This progression was abolished by treatment with PETN (6 mg/kg bw/day) suggesting a vasoprotective activity of the NO donor PETN in experimental atherosclerosis (reprinted from Ref. [78] with permission).

glyceryl trinitrate patch with enalapril showed a significantly greater reduction in mortality than enalapril alone [82].

Although there are some limitations in both of these trials such as nitrate use in the control groups, it seems likely that the effects of nitrates on mortality after myocardial infarction are lower than previously reported. One explanation for the discrepancy between earlier investigations and the mentioned mega-trials could be that the latter studies were carried out after establishment of the benefits of thrombolysis with streptokinase [84]. Another important point is that nitrate therapy was given only for 4–6 weeks, a period which might have been too short to evaluate the inhibitory effects on the progression of atherosclerosis. For example, statins such as simvastatin show beneficial effects on mortality after a treatment period of at least 6 months [85]. Thus, it remains an open question whether organic nitrates can delay the development of atherosclerosis and increase the life expectancy of patients with active atherosclerosis.

4.2. Direct activators of soluble guanylate cyclase

The compound YC-1, a benzylindazol derivative, was reported to be an activator of sGC leading to an increase of intracellular cGMP [86]. YC-1 can stimulate sGC independent of NO but can also potentiate the NO-induced

stimulation, presumably by stabilization of the enzyme's active configuration [87,88]. YC-1 is ineffective in patients because it is not a very potent (NO-independent) stimulator of sGC. However, the existence of this compound strongly stimulated research activities in the pharmaceutical industry in order to find new drugs based on the chemical structure of YC-1. These research activities resulted in the discovery of a series of new compounds which can stimulate sGC independent of NO, are much more potent than YC-1 and display useful pharmacological actions such as inhibition of platelet aggregation and vasodilation [89]. Further investigations on the mechanism of action showed that there is a new regulatory site on the sGC: the region of the cysteines 238 and 243 in the α_1 -subunit of the enzyme [90]. The value of these drugs in pharmacotherapy remains to be elucidated.

4.3. L-Arginine

The amino acid L-arginine is the substrate of eNOS and is converted to citrulline and NO. Administration of L-arginine has been shown to reduce atherosclerosis and endothelial dysfunction in cholesterol-fed rabbits [91]. Subsequent clinical studies demonstrated that L-arginine improves endothelium-dependent vasodilation in hypercholesterolemic humans [92]. It is believed that the beneficial effects of L-arginine administration are partially caused by a competition between this genuine amino acid and the derivative asymmetric dimethyl L-arginine (ADMA) which is an endogenous inhibitor of eNOS activity [93,94]. However, it should be noted that high doses of L-arginine, which increase the plasma concentrations by 20-fold, can exert a variety of unspecific effects such as increases in plasma insulin, prolactin and glucagon in healthy subjects and in patients with diabetes or essential hypertension [95]. The overall clinical pharmacology of L-arginine has been reviewed recently [96]. Accordingly, it seems possible that the administration of L-arginine can improve clinical symptoms of cardiovascular diseases such as peripheral arterial disease, coronary artery disease and congestive heart failure.

4.4. Phosphodiesterase inhibitors

By catalyzing cyclic nucleotide hydrolysis, the PDEs are important determinants regulating the intracellular concentrations and the biological actions of cAMP and cGMP [97]. Intense biochemical and molecular genetic research has revealed the complexity and diversity of the PDE superfamily which contains at least 11 highly regulated and structurally-related gene families [15]. These PDE isoforms are often tissue-specific and differ in their sensitivity to specific inhibitors, subcellular distribution, mechanism of regulation and substrate selectivity (Table 1) [97]. The pharmacotherapeutic principle of isoform-selective PDE inhibition is the subject of intensive research in many different fields including asthma, allergies, diabetes, restenosis and arthritis [98]. The importance of cyclic nucleotide signaling and the molecular diversity of PDEs make these proteins interesting targets for selective intervention including modulation of the NO–cGMP pathway.

Sildenafil is an inhibitor of the cGMP-specific PDE V and has been shown to be effective in the treatment of erectile dysfunction by elevating cGMP levels in the smooth muscle cells of the corpus cavernosum [99]. PDE V is the most important enzyme for cGMP metabolism in this type of smooth muscle while smooth muscle cells of the systemic circulation contain PDE IAI as another important cGMP-specific PDE [100]. There is evidence indicating that the increase in erectile function induced by sildenafil involves the cGMP-dependent inhibition of PDE III and subsequent elevation of cAMP levels in the corpus cavernosum [101]. Inhibition of PDE V by sildenafil can induce severe hypotension when combined with NO-donating drugs such as nitrates but under resting conditions sildenafil has minor effects on the vasculature as indicated by a lack of hemodynamic actions [102]. Theoretically, selective PDE V inhibitors may have a potential to exert protective actions such as prevention of overt erectile dysfunction but so far evidence for this is lacking.

4.5. Other drugs

A variety of drugs can indirectly affect endogenous NO

Table 1
Pharmacological inhibition of cGMP-metabolizing phosphodiesterase isoforms

Isoform	Nucleotides	Regulation	Inhibitors
PDE IAI	cGMP \gg cAMP	Ca ²⁺ /Calmodulin-sensitive	8-Methoxymethyl-IBMX, Vinpocetine
PDE II	cAMP \gg cGMP	cGMP-stimulated	EHNA
PDE III	cAMP \gg cGMP	cGMP-inhibited	Trequinsin, Quazinson, Amrinone, Milrinone, Cilostazol, Cilostamide
PDE V	cGMP-specific		Sildenafil, zaprinast, vardenafil
PDE VI	cGMP-specific		Sildenafil, zaprinast, vardenafil
PDE IX	cGMP-specific		Unknown
PDE X	cGMP \gg cAMP	cAMP-inhibited	Unknown
PDE XI	cAMP, cGMP		Unknown

IBMX, isobutylmethylxanthine; EHNA, *erythro*-9-(2-hydroxy-3-nonyl)-adenine.

production [12]. Inhibitors of cholesterol synthesis (statins) such as lovastatin have been shown to increase the expression of eNOS [103]. Although statins act predominantly by reducing plasma cholesterol levels, it seems possible that other effects of these drugs such as stimulation of endothelial NO production contribute to their beneficial action in coronary artery disease [104].

Another group of drugs showing activation of endothelial NO production are angiotensin converting enzyme inhibitors such as captopril [105,106]. Inhibition of the carboxypeptidase (angiotensin) converting enzyme results in both reduced formation of angiotensin II from angiotensin I and reduced breakdown of bradykinin, a potent stimulator of endothelial NO production. Converting enzyme inhibitors can increase vascular cGMP levels and induce NO dependent vasodilation mediated by endothelium-derived kinins [105]. These findings are consistent with a recent clinical study which showed that bradykinin contributes to the blood pressure-reducing effects of captopril [106]. Large clinical trials demonstrated that converting enzyme inhibitors reduce the mortality of patients with acute myocardial infarction or heart failure [82,83,107]. Again, it is tempting to speculate that the increase of vascular NO levels during treatment with converting enzyme inhibitors contributes to the beneficial effects on mortality and morbidity of cardiovascular patients.

Finally, it was demonstrated that the β -blocker nebivolol can elicit endothelium-dependent vasodilation in human forearm vasculature and the hand veins [108,109]. There is evidence that augmentation of vascular NO release is not mediated by nebivolol itself but rather by an unknown metabolite which can activate endothelial β_2 -adrenoceptors and initiate a substantial rise in endothelial free Ca^{2+} [110]. It is not known whether nebivolol exerts vasoprotective effects in animal models of atherosclerosis or improves the mortality of patients with coronary artery disease.

References

- [1] Moncada S, Higgs A. Mechanisms of disease: the L-arginine-nitric oxide pathway. *New Engl J Med* 1993;329:2002–2012.
- [2] Busse R, Fleming I. Endothelial dysfunction in atherosclerosis. *J Vasc Res* 1996;33:181–194.
- [3] Kojda G, Harrison DG. Interactions between NO and reactive oxygen species: Pathophysiological importance in atherosclerosis, hypertension, diabetes and heart failure. *Cardiovasc Res* 1999;43:562–571.
- [4] Schachinger V, Britten MB, Zeiher AM. Prognostic impact of coronary vasodilator dysfunction on adverse long-term outcome of coronary heart disease. *Circulation* 2000;101:1899–1906.
- [5] Lucas KA, Pitari GM, Kazerooni S et al. Guanylyl cyclases and signaling by cyclic GMP. *Pharmacol Rev* 2000;52:375–414.
- [6] Rajfer J, Aronson WJ, Bush PA, Dorey FJ, Ignarro LJ. Nitric oxide as a mediator of relaxation of the corpus cavernosum in response to nonadrenergic, noncholinergic neurotransmission. *New Engl J Med* 1992;326:90–94.
- [7] Green SJ, Scheller LF, Marletta MA et al. Nitric oxide: cytokine-regulation of nitric oxide in host resistance to intracellular pathogens. *Immunol Lett* 1994;43:87–94.
- [8] Wilson RI, Yanovsky J, Gödecke A, Stevens DR, Schrader J, Haas HL. Endothelial nitric oxide synthase and LTP. *Nature* 1997;386:338.
- [9] Pohl U, Holtz J, Busse R, Bassenge E. Crucial role of endothelium in the vascular response to increased flow in vivo. *Hypertension* 1986;8:37–44.
- [10] Dimmeler S, Fleming I, Fisslthaler B et al. Activation of nitric oxide synthase in endothelial cells by Akt-dependent phosphorylation. *Nature* 1999;399:601–605.
- [11] Luo Z, Fujio Y, Kureishi Y et al. Acute modulation of endothelial Akt/PKB activity alters nitric oxide-dependent vasomotor activity in vivo. *J Clin Invest* 2000;106:493–499.
- [12] Ignarro LJ, Cirino G, Casini A, Napoli C. Nitric oxide as a signaling molecule in the vascular system: an overview. *J Cardiovasc Pharmacol* 1999;34:879–886.
- [13] Pfeifer A, Klatt P, Massberg S et al. Defective smooth muscle regulation in cGMP kinase I-deficient mice. *EMBO J* 1998;17:3045–3051.
- [14] Massberg S, Sausbier M, Klatt P et al. Increased adhesion and aggregation of platelets lacking cyclic guanosine 3',5'-monophosphate kinase I. *J Exp Med* 1999;189:1255–1263.
- [15] Soderling SH, Beavo JA. Regulation of cAMP and cGMP signaling: new phosphodiesterases and new functions [in process citation]. *Curr Opin Cell Biol* 2000;12:174–179.
- [16] Furchgott RF, Zawadzki JV. The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. *Nature* 1980;288:373–376.
- [17] Rossi GP, Seccia TM, Nussdorfer GG. Reciprocal regulation of endothelin-1 and nitric oxide: relevance in the physiology and pathology of the cardiovascular system. *Int Rev Cytol* 2001;209:241–272.
- [18] Stamler JS, Loh E, Roddy M-A, Currie KE, Creager MA. Nitric oxide regulates basal systemic and pulmonary vascular resistance in healthy humans. *Circulation* 1994;89:2035–2040.
- [19] Huang PL, Huang ZH, Mashimo H et al. Hypertension in mice lacking the gene for endothelial nitric oxide synthase. *Nature* 1995;377:239–242.
- [20] Kannel WB. Fifty years of Framingham Study contributions to understanding hypertension. *J Hum Hypertens* 2000;14:83–90.
- [21] Cornwell TL, Pryzwansky KB, Wyatt TA, Lincoln TM. Regulation of sarcoplasmic reticulum protein phosphorylation by localized cyclic GMP-dependent protein kinase in vascular smooth muscle cells. *Mol Pharmacol* 1991;40:923–931.
- [22] Simmerman HK, Jones LR. Phospholamban: protein structure, mechanism of action, and role in cardiac function. *Physiol Rev* 1998;78:921–947.
- [23] Cohen RA, Weisbrod RM, Gericke M et al. Mechanism of nitric oxide-induced vasodilation—Refilling of intracellular stores by sarcoplasmic reticulum Ca^{2+} ATPase and inhibition of store-operated Ca^{2+} influx. *Circ Res* 1999;84:210–219.
- [24] Schlossmann J, Ammendola A, Ashman K et al. Regulation of intracellular calcium by a signalling complex of IRAG, IP3 receptor and cGMP kinase β . *Nature* 2000;404:197–201.
- [25] Bolotina VM, Najibi S, Palacino JJ, Pagano PJ, Cohen RA. Nitric oxide directly activates calcium-dependent potassium channels in vascular smooth muscle. *Nature* 1994;368:850–853.
- [26] Sausbier M, Schubert R, Voigt V et al. Mechanisms of NO/cGMP-dependent vasorelaxation [in process citation]. *Circ Res* 2000;87:825–830.
- [27] Ishikawa T, Hume JR, Keef KD. Regulation of Ca^{2+} channels by cAMP and cGMP in vascular smooth muscle cells. *Circ Res* 1993;73:1128–1137.
- [28] Horowitz A, Menice CB, Laporte R, Morgan KG. Mechanisms of smooth muscle contraction. *Physiol Rev* 1996;76:967–1003.

- [29] Radomski MW, Moncada S. Regulation of vascular hemostasis by nitric oxide. *Thromb Haemost* 1993;70:36–41.
- [30] Benjamin N, Dutton JAE, Ritter JM. Human vascular smooth muscle cells inhibit platelet aggregation when incubated with glyceryl trinitrate: evidence for generation of nitric oxide. *Br J Pharmacol* 1991;102:847–850.
- [31] Moro MA, Russell RJ, Cellick S et al. cGMP mediates the vascular and platelet actions of nitric oxide: Confirmation using an inhibitor of the soluble guanylyl cyclase. *Proc Natl Acad Sci USA* 1996;93:1480–1485.
- [32] Rao GH, Krishnamurthi S, Raji L, White JG. Influence of nitric oxide on agonist-mediated calcium mobilization in platelets. *Biochem Med Metab Biol* 1990;43:271–275.
- [33] Nguyen BL, Saitoh M, Ware JA. Interaction of nitric oxide and cGMP with signal transduction in activated platelets. *Am J Physiol Heart Circ Physiol* 1991;261:H1043–H1052.
- [34] Trepakova ES, Cohen RA, Bolotina VM. Nitric oxide inhibits capacitative cation influx in human platelets by promoting sarcoplasmic/endoplasmic reticulum Ca^{2+} -ATPase-dependent refilling of Ca^{2+} stores. *Circ Res* 1999;84:201–209.
- [35] Bowen R, Haslam RJ. Effects of nitrovasodilators on platelet cyclic nucleotide levels in rabbit blood; role for cyclic AMP in synergistic inhibition of platelet function by $SiN-1$ and prostaglandin E_1 . *J Cardiovasc Pharmacol* 1991;17:424–433.
- [36] Fischer TH, White GC. Partial purification and characterization of thrombolamban, a 22 000 dalton cAMP-dependent protein kinase substrate in platelets. *Biochem Biophys Res Commun* 1987;149:700–706.
- [37] Geiger J, Nolte C, Walter U. Regulation of calcium mobilization and entry in human platelets by endothelium-derived factors. *Am J Physiol Cell Physiol* 1994;267:C236–C244.
- [38] Ross R. Mechanisms of disease—atherosclerosis—An inflammatory disease. *New Engl J Med* 1999;340:115–126.
- [39] Kunsch C, Medford RM. Oxidative stress as a regulator of gene expression in the vasculature. *Circ Res* 1999;85:753–766.
- [40] Chen F, Castranova V, Shi X, Demers LM. New insights into the role of nuclear factor- κB , a ubiquitous transcription factor in the initiation of diseases. *Clin Chem* 1999;45:7–17.
- [41] Ross R. Atherosclerosis—an inflammatory disease. *New Engl J Med* 1999;340:115–126.
- [42] Kubes P, Suzuki M, Granger DN. Nitric oxide: An endogenous modulator of leukocyte adhesion. *Proc Natl Acad Sci USA* 1991;88:4651–4655.
- [43] Khan BV, Harrison DG, Olbrych MT, Alexander RW, Medford RM. Nitric oxide regulates vascular cell adhesion molecule 1 gene expression and redox-sensitive transcriptional events in human vascular endothelial cells. *Proc Natl Acad Sci USA* 1996;93:9114–9119.
- [44] Niu XF, Smith CW, Kubes P. Intracellular oxidative stress induced by nitric oxide synthesis inhibition increases endothelial cell adhesion to neutrophils. *Circ Res* 1994;74:1133–1140.
- [45] Schwartz SM. Smooth muscle migration in atherosclerosis and restenosis. *J Clin Invest* 1997;99:2814–2817.
- [46] Berk BC. Vascular smooth muscle growth: autocrine growth mechanisms. *Physiol Rev* 2001;81:999–1030.
- [47] Scott-Burden T, Vanhoutte PM. The endothelium as a regulator of vascular smooth muscle proliferation. *Circulation* 1993;87(suppl 5):51–55.
- [48] Nakaki T, Nakayama M, Kato R. Inhibition by nitric oxide and nitric oxide-producing vasodilators of DNA synthesis in vascular smooth muscle cells. *Eur J Pharmacol Mol Pharmacol* 1990;189:347–353.
- [49] Moroi M, Zhang L, Yasuda T et al. Interaction of genetic deficiency of endothelial nitric oxide, gender, and pregnancy in vascular response to injury in mice. *J Clin Invest* 1998;101:1225–1232.
- [50] Cornwell TL, Arnold E, Boerth NJ, Lincoln TM. Inhibition of smooth muscle cell growth by nitric oxide and activation of cAMP-dependent protein kinase by cGMP. *Am J Physiol Cell Physiol* 1994;267:C1405–C1413.
- [51] Osinski MT, Rauch BH, Schror K. Antimitogenic actions of organic nitrates are potentiated by sildenafil and mediated via activation of protein kinase A. *Mol Pharmacol* 2001;59:1044–1050.
- [52] Kojda G, Kottenberg K, Nix P et al. Low increase in cGMP induced by organic nitrates and nitrovasodilators improves contractile response of rat ventricular myocytes. *Circ Res* 1996;78:91–101.
- [53] Tanner FC, Meier P, Greutert H, Champion C, Nabel EG, Lüscher TF. Nitric oxide modulates expression of cell cycle regulatory proteins—a cytostatic strategy for inhibition of human vascular smooth muscle cell proliferation. *Circulation* 2000;101:1982–1989.
- [54] Cook SJ, McCormick F. Inhibition by cAMP of Ras-dependent activation of Raf [see comments]. *Science* 1993;262:1069–1072.
- [55] Ignarro LJ, Buga GM, Wei LH et al. Role of the arginine–nitric oxide pathway in the regulation of vascular smooth muscle cell proliferation. *Proc Natl Acad Sci USA* 2001;98:4202–4208.
- [56] Wolin MS. Interactions of oxidants with vascular signaling systems. *Arterioscler Thromb Vasc Biol* 2000;20:1430–1442.
- [57] O'Donnell VB, Freeman BA. Interactions between nitric oxide and lipid oxidation pathways—Implications for vascular disease. *Circ Res* 2001;88:12–21.
- [58] Koppenol WH. The basic chemistry of nitrogen monoxide and peroxynitrite. *Free Radic Biol Med* 1998;25:385–391.
- [59] Bonini MG, Radi R, Ferrer-Sueta G, Ferreira AM, Augusto O. Direct EPR detection of the carbonate radical anion produced from peroxynitrite and carbon dioxide. *J Biol Chem* 1999;274:10802–10806.
- [60] Bonini MG, Augusto O. Carbon dioxide stimulates the production of thiyl, sulfinyl, and disulfide radical anion from thiol oxidation by peroxynitrite. *J Biol Chem* 2001;276:9749–9754.
- [61] Pfeiffer S, Schmidt K, Mayer B. Dityrosine formation outcompetes tyrosine nitration at low steady-state concentrations of peroxynitrite—Implications for tyrosine modification by nitric oxide/superoxide in vivo. *J Biol Chem* 2000;275:6346–6352.
- [62] Radi R, Beckman JS, Bush KM, Freeman BA. Peroxynitrite oxidation of sulfhydryls. The cytotoxic potential of superoxide and nitric oxide. *J Biol Chem* 1991;266:4244–4250.
- [63] Beckman JS, Koppenol WH. Nitric oxide, superoxide, and peroxynitrite: the good, the bad, and the ugly. *Am J Physiol* 1999;271:C1424–C1437.
- [64] Recalcati S, Taramelli D, Conte D, Cairo G. Nitric oxide-mediated induction of ferritin synthesis in J774 macrophages by inflammatory cytokines: role of selective iron regulatory protein-2 downregulation. *Blood* 1998;91:1059–1066.
- [65] Oberle S, Schwartz P, Abate A, Schroder H. The antioxidant defense protein ferritin is a novel and specific target for pentaerythritol tetranitrate in endothelial cells. *Biochem Biophys Res Commun* 1999;261:28–34.
- [66] Balla G, Jacob HS, Balla J et al. Ferritin: a cytoprotective antioxidant stratagem of endothelium. *J Biol Chem* 1992;267:18148–18153.
- [67] Durante W, Kroll MH, Christodoulides N, Peyton KJ, Schafer AI. Nitric oxide induces heme oxygenase-1 gene expression and carbon monoxide production in vascular smooth muscle cells. *Circ Res* 1997;80:557–564.
- [68] Maines MD. The heme oxygenase system: a regulator of second messenger gases. *Annu Rev Pharmacol Toxicol* 1997;37:517–554.
- [69] Stocker R, Yamamoto Y, McDonagh AF, Glazer AN, Ames BN. Bilirubin is an antioxidant of possible physiological importance. *Science* 1987;235:1043–1046.
- [70] Ishikawa K, Sugawara D, Wang X et al. Heme oxygenase-1 inhibits atherosclerotic lesion formation in LDL-receptor knockout mice. *Circ Res* 2001;88:506–512.
- [71] Fukui T, Siegfried MR, Ushio-Fukai M et al. Regulation of the vascular extracellular superoxide dismutase by nitric oxide and exercise training. *J Clin Invest* 2000;105:1631–1639.

- [72] Ström P, Karlsson K, Johansson BO, Marklund SL. The interstitium of the human arterial wall contains very large amounts of extracellular superoxide dismutase. *Arterioscler Thromb Vasc Biol* 1995;15:2032–2036.
- [73] Drummond GR, Cai H, Davis ME, Ramasamy S, Harrison DG. Transcriptional and posttranscriptional regulation of endothelial nitric oxide synthase expression by hydrogen peroxide. *Circ Res* 2000;86:347–354.
- [74] Kojda G, Cheng YC, Burchfield J, Harrison DG. Dysfunctional regulation of endothelial nitric oxide synthase (eNOS) expression in response to exercise in mice lacking one eNOS gene. *Circulation* 2001;103:2839–2844.
- [75] Kojda G. Therapeutic importance of nitrovasodilators. In: Mayer B, editor. *Handbook of pharmacology. Nitric oxide*, vol. 143, Berlin, New York, Tokyo: Springer Verlag, 2000, pp. 365–384.
- [76] Minamiyama Y, Takemura S, Akiyama T et al. Isoforms of cytochrome P450 on organic nitrate-derived nitric oxide release in human heart vessels. *FEBS Lett* 1999;452:165–169.
- [77] Kojda G, Stein D, Kottenberg F, Schnaith EM, Noack E. In vivo effects of pentaerythritol-tetranitrate and isosorbide-5-mononitrate on the development of atherosclerosis and endothelial dysfunction in cholesterol-fed rabbits. *J Cardiovasc Pharmacol* 1995;25:763–773.
- [78] Hacker A, Müller S, Meyer W, Kojda G. The nitric oxide donor pentaerythritol tetranitrate can preserve endothelial function in established atherosclerosis. *Br J Pharmacol* 2001;132:1707–1714.
- [79] Müller S, Meyer W, Kojda G. Vasoprotection by ISMN in experimental atherosclerosis. *Basic Res Cardiol* 2001;96(suppl 1):55, abstract.
- [80] Bult H, De Meyer GRY, Herman AG. Influence of chronic treatment with a nitric oxide donor on fatty streak development and reactivity of the rabbit aorta. *Br J Pharmacol* 1995;114:1371–1382.
- [81] Yusuf S, MacMahon S, Collins R, Peto R. Effect of intravenous nitrates on mortality in acute myocardial infarction: an overview of the randomised trials. *Lancet* 1988;1(8594):1088–1092.
- [82] Gruppo Italiano per lo Studio della Sopravvivenza nell'Infarto Miocardico. GISSI-3: effects of lisinopril and transdermal glyceryl trinitrate singly and together on 6-week mortality and ventricular function after acute myocardial infarction. *Lancet* 1994;343:1115–1122.
- [83] ISIS-4 Collaborative Group. ISIS-4: A randomised factorial trial assessing early oral captopril, oral mononitrate, and intravenous magnesium sulphate in 58 050 patients with suspected acute myocardial infarction. *Lancet* 1995;345:669–685.
- [84] ISIS-2 (Second International Study of Infarct Survival) Collaborative Group. Randomised trial of intravenous streptokinase, oral aspirin, both, or neither among 17 187 cases of suspected acute myocardial infarction: ISIS-2. *Lancet* 1988;2(8607):349–360.
- [85] Anonymous. Randomised trial of cholesterol lowering in 4444 patients with coronary heart disease: the scandinavian simvastatin survival study (4s) [see comments]. *Lancet* 1994;344:1383–1389.
- [86] Ko F-N, Wu C-C, Kuo S-C, Lee F-Y, Teng C-M. YC-1, a novel activator of platelet guanylate cyclase. *Blood* 1994;84:4226–4233.
- [87] Wu CC, Ko FN, Kuo SC, Lee FY, Teng CM. YC-1 inhibited human platelet aggregation through NO-independent activation of soluble guanylate cyclase. *Br J Pharmacol* 1995;116:1973–1978.
- [88] Friebe A, Schultz G, Koesling D. Sensitizing soluble guanylyl cyclase to become a highly CO-sensitive enzyme. *EMBO J* 1996;15:6863–6868.
- [89] Straub A, Stasch JP, Alonso-Alija C et al. NO-independent stimulators of soluble guanylate cyclase. *Bioorg Med Chem Lett* 2001;11:781–784.
- [90] Stasch JP, Becker EM, Alonso-Alija C et al. NO-independent regulatory site on soluble guanylate cyclase. *Nature* 2001;410:212–215.
- [91] Cooke JP, Singer AH, Tsao P et al. Antiatherogenic effects of L-arginine in the hypercholesterolemic rabbit. *J Clin Invest* 1992;90:1168–1172.
- [92] Creager MA, Gallagher SJ, Girerd XJ et al. L-Arginine improves endothelium-dependent vasodilation in hypercholesterolemic humans. *J Clin Invest* 1992;90:1248–1253.
- [93] Vallance P, Leone A, Calver A, Collier J, Moncada S. Accumulation of an endogenous inhibitor of nitric oxide synthesis in chronic renal failure. *Lancet* 1992;339:572–575.
- [94] Bode-Böger SM, Böger RH, Kienke S, Junker W, Frölich JC. Elevated L-arginine/dimethylarginine ratio contributes to enhanced systemic NO production by dietary L-arginine in hypercholesterolemic rabbits. *Biochem Biophys Res Commun* 1996;219:598–603.
- [95] MacAllister RJ, Calver AL, Collier J et al. Vascular and hormonal responses to arginine: provision of substrate for nitric oxide or nonspecific effect? *Clin Sci (Lond)* 1995;89:183–190.
- [96] Boger RH, Bode-Böger SM. The clinical pharmacology of L-arginine. *Annu Rev Pharmacol Toxicol* 2001;41:79–99.
- [97] Manganelli VC, Degerman E. Cyclic nucleotide phosphodiesterases (PDEs): diverse regulators of cyclic nucleotide signals and inviting molecular targets for novel therapeutic agents. *Thromb Haemost* 1999;82:407–411.
- [98] Polson JB, Strada SJ. Cyclic nucleotide phosphodiesterases and vascular smooth muscle. *Annu Rev Pharmacol Toxicol* 1996;36:403–427.
- [99] Boolell M, Allen MJ, Ballard SA et al. Sildenafil: an orally active type 5 cyclic GMP-specific phosphodiesterase inhibitor for the treatment of penile erectile dysfunction. *Int J Impot Res* 1996;8:47–52.
- [100] Sonnenburg WK, Seger D, Kwak KS et al. Identification of inhibitory and calmodulin-binding domains of the PDE1A1 and PDE1A2 calmodulin-stimulated cyclic nucleotide phosphodiesterases. *J Biol Chem* 1995;270:30989–31000.
- [101] Stief CG, Uckert S, Becker AJ et al. Effects of sildenafil on cAMP and cGMP levels in isolated human cavernous and cardiac tissue. *Urology* 2000;55:146–150.
- [102] Hermann HC, Chang G, Klugherz BD, Mahoney PD. Hemodynamic effects of sildenafil in men with severe coronary artery disease. *New Engl J Med* 2000;342:1622–1626.
- [103] Laufs U, La Fata V, Plutzky J, Liao JK. Upregulation of endothelial nitric oxide synthase by HMG CoA reductase inhibitors. *Circulation* 1998;97:1129–1135.
- [104] The Long-Term Intervention with Pravastatin in Ischaemic Disease (LIPID) Study Group. Prevention of cardiovascular events and death with pravastatin in patients with coronary heart disease and a broad range of initial cholesterol levels [see comments]. *New Engl J Med* 1998;339:1349–1357.
- [105] Hecker M, Barn AT, Busse R. Relaxation of isolated coronary arteries by angiotensin-converting enzyme inhibitors: Role of endothelium-derived kinins. *J Vasc Res* 1993;30:257–262.
- [106] Gainer JV, Morrow JD, Loveland A, King DJ, Brown NJ. Effect of bradykinin-receptor blockade on the response to angiotensin-converting-enzyme inhibitor in normotensive and hypertensive subjects. *New Engl J Med* 1998;339:1285–1292.
- [107] The SOLVD Investigators. Effect of enalapril on survival in patients with reduced left ventricular ejection fractions and congestive heart failure. *New Engl J Med* 1991;325:293–302.
- [108] Bowman AJ, Chen CP, Ford GA. Nitric oxide mediated venodilator effects of nebivolol. *Br J Clin Pharmacol* 1994;38:199–204.
- [109] Cockcroft JR, Chowieńczyk PJ, Brett SE et al. Nebivolol vasodilates human forearm vasculature: evidence for an L-arginine–NO-dependent mechanism. *J Pharmacol Exp Ther* 1995;274:1067–1071.
- [110] Broeders MAW, Doevendans PA, Bekkers BCAM et al. Nebivolol: a third-generation β -blocker that augments vascular nitric oxide release endothelial β_2 -adrenergic receptor-mediated nitric oxide production. *Circulation* 2000;102:677–684.
- [111] Hambrecht R, Wolf A, Gielen S et al. Effect of exercise on coronary endothelial function in patients with coronary artery disease [see comments]. *New Engl J Med* 2000;342:454–460.